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CONCERNING A FILING UNDER 35 U.S.C. 371

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INTERNATIONAL APPLICATION NO.

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PRIORITY DATE CLAIMED

26 February 1999 (26.2.99)

TITLE OF INVENTION

USE OF COLLAGENASE IN THE PREPARATION OF NEURAL STEM CELL CULTURES

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☐ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Express Mail Label No: EL390884433US

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 09/890539		INTERNATIONAL APPLICATION NO. PCT/US/00/05402		ATTORNEY'S DOCKET NUMBER 17810-509	
24. The following fees are submitted:				CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00					
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	57 - 20 =	37	x \$18.00	\$666.00	
Independent claims	4 - 3 =	1	x \$80.00	\$80.00	
Multiple Dependent Claims (check if applicable).				<input checked="" type="checkbox"/>	\$270.00
TOTAL OF ABOVE CALCULATIONS =				\$2,006.00	
<input type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				\$0.00	
SUBTOTAL =				\$2,006.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$0.00	
TOTAL NATIONAL FEE =				\$2,006.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).				<input type="checkbox"/>	\$0.00
TOTAL FEES ENCLOSED =				\$2,006.00	
				Amount to be refunded	\$
				charged	\$

- a. ☒ A check in the amount of **\$2,006.00** to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0311**. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

31 July 2001 (31.07.01)

DATE

USE OF COLLAGENASE IN THE PREPARATION OF NEURAL STEM CELL CULTURES

CLAIM OF PRIORITY

This invention claims priority to United States patent application 09/258,529, filed February 26, 1999.

TECHNICAL FIELD

This invention relates generally to a production or manufacturing process for the *in vitro* proliferation and culture of neural stem cell cultures using collagenase to dissociate the cultured cells.

BACKGROUND OF THE INVENTION

The development of the mammalian central nervous system (CNS) begins in the early stage of fetal development and continues until the post-natal period. The mature mammalian CNS is composed of neuronal cells (neurons), and glial cells (astrocytes and oligodendrocytes). The first step in neural development is cell birth, which is the precise temporal and spatial sequence in which stem cells and stem cell progeny (*i.e.* daughter stem cells and progenitor cells) proliferate.

One identifying feature of a stem cell is its ability to exhibit self-renewal or to generate more of itself. A definition of a stem cell is provided by Potten & Loeffler, 110 Development 1001 (1990) who have defined stem cells as "undifferentiated cells capable of (a) proliferation, (b) self-maintenance, (c) the production of a large number of differentiated functional progeny, (d) regenerating the tissue after injury, and (e) a flexibility in the use of these options." The role of stem cells is to replace cells that are lost by natural cell death, injury or disease.

United States patents 5,750,376, 5,851,832 (both naming Weiss) and 5,753,506 (Johe), each incorporated herein by reference, refer to *in vitro* cultures containing neural stem cells. The Weiss patents refer to both suspension and adherent culture, while Johe refers to particular adherent cultures. When the cells are propagated as neurospheres in suspension culture, within 3-4 days in the presence of a proliferation-inducing growth factor, a multipotent neural stem cell begins to divide giving rise to a cluster of undifferentiated cells referred to as a "neurosphere". The cells of a single neurosphere are clonal in nature because they are the progeny of a single

neural stem cell. In the continued presence of one or more proliferation-inducing growth factors, such as EGF, bFGF, or the like (and combinations thereof), cells within the neurosphere continue to divide resulting in an increase in the size of the neurosphere and the number of undifferentiated cells. The cells within the neurosphere are immunoreactive for nestin, an intermediate filament protein found in many types of undifferentiated CNS cells. In contrast, mature differentiated cell types derived from the neural stem cell progeny are predominantly negative for nestin.

In the prior art, the cells in the cluster were mechanically dissociated by trituration to produce single cells between passages. Trituration, because it is a mechanical process, exerts shear forces on the cells, that may reduce cell viability between passages. The object of the present invention is to provide an improved culture and manufacture process that increases cell viability from passage to passage and maintains more of the most primitive cells (with the greatest differentiation potential and self-renewal capability).

SUMMARY OF THE INVENTION

The invention provides a method for the *in vitro* proliferation of multipotent neural stem cell culture using collagenase to dissociate cells in neurospheres between passages. According to the method of this invention, use of collagenase results in improved neural stem cell culture viability, increased number of proliferated cells in those cultures over time, and improved maintenance of cell cultures, as compared with dissociation by trituration or other enzymatic treatments such as trypsinization.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows a comparison between the prior art trituration method of dissociating proliferating human neurospheres and the collagenase method of the invention, demonstrating an increased number of viable cells over time using the collagenase method.

DETAILED DESCRIPTION OF THE INVENTION

Introduction. The invention provides a novel manufacturing process for proliferating neural stem cell cultures, using collagenase to dissociate neurospheres ("aggregated" cells). This method results in an unexpectedly improved neural stem cell culture viability and increased

number of proliferated cells over time, as compared with prior art trituration and trypsinization methods for dissociating neural stem cell cultures.

In one embodiment of the collagenase manufacture process, neurosphere cells are harvested and centrifuged (e.g., at 1000 rpm for 3-5 min). After aspirating medium, neurospheres are resuspended in collagenase solution (e.g., 1 ml of pre-warmed (37°C) 0.5 mg/ml), and incubated with the collagenase. After the incubation, cell suspension is diluted in medium and cultured.

In the prior art trituration method, neurosphere cells were harvested and centrifuged. After aspirating medium, neurospheres were resuspended in ~200 µl of medium. Neurospheres were tritured by using a pipette (e.g., a P200 pipetman with 75 µl volumes, about 100 times). Then, the cell suspension was diluted in medium and cultured.

Collagenase. Any collagenase which is effective for dissociating neural stem cells in culture may be used in the manufacture process of the invention. "Collagenase" is an enzyme that digests the extracellular matrix protein collagen (Harper, 49 Ann. Rev. Biochem. 1063 (1980)). One source of collagenase is the bacterium *Clostridium histolyticum*. One collagenase assay is a modification of Mandl *et al.*, 32 J. Clin. Invest. 1323 (1953), whereby collagenase is incubated for 5 hr with native collagen. The extent of collagen breakdown is determined using the colorimetric ninhydrin method of Moore & Stein, 176 Biol. Chem 367 (1948). For collagenase unit definition, 1 unit releases one µmole of L-leucine equivalents from collagen in 5 hr at 37°C, pH 7.5.

Crude collagenases can be used for cell dissociation procedures. Crude collagenase preparations contain not only several collagenases but also a sulfhydryl protease, clostripain, a trypsin-like enzyme, and an aminopeptidase. In some embodiments, we prefer crude collagenase preparations, because of the presence of these additional activities. This combination of collagenolytic and proteolytic activities is effective at breaking down intercellular matrices, the essential part of tissue dissociation. Crude collagenase is inhibited by metal chelating agents such as cysteine, EDTA or o-phenanthroline. It is also inhibited by alpha-2-macroglobulin, a large plasma glycoprotein. Ca²⁺ is thought to be required for enzyme activity.

Commercially-available sources of collagenase are useful in the methods of this invention. For example, purified collagenase contains minimal secondary proteolytic activities, but with high collagenase activity. Purified collagenase can be collagenase H (Cat # 1 087 789) from Boehringer Mannheim (Indianapolis, IN). A stock solution of 0.5 mg/ml collagenase is prepared in DPBS containing 0.1 % BSA, and stored -20°C. Other commercially available sources

are Dispase (Boehringer Mannheim), Liberase (Boehringer Mannheim) or collagenase (Serva). The range of collagenase used can be from 100-1000 µg/ml (18-180 mU/ml), preferably between 300 - 700 µg/ml, (54-126 mU/ml) most preferably about 500 µg/ml (90 mU/ml).

Isolation and In Vitro Proliferation of Multipotent Self-renewing CNS Neural Stem Cells

Neurobiologists have used various terms interchangeably to describe the undifferentiated cells of the CNS. Terms such as "stem cell", "precursor cell", and progenitor cell" were once used in the scientific literature. However, there are different types of undifferentiated neural cells, with differing characteristics and fates. The terminology used for undifferentiated multipotent neural cells has evolved such that these cells are now termed "neural stem cells" United States patent 5,750,376 defines the "neural stem" cell proliferated *in vitro* to mean "an oligopotent or multipotent stem cell which is able to divide without limit and under specific conditions can produce daughter cells which terminally differentiate into neurons and glia." The capability of a cell to divide without limit and produce daughter cells which terminally differentiate into neurons and glia are CNS stem cell characteristics. A CNS neural stem cell is capable of self maintenance, meaning that with each cell division, one daughter cell will also be a stem cell. A CNS neural stem cell can be induced to proliferate using the methods of the present invention.

The non-stem cell progeny of a neural stem cell may include progenitor cells. The progenitor cells generated from a single multipotent self-renewing CNS neural stem cell are capable of differentiating into neurons, astrocytes (type I and type II) or oligodendrocytes. By contrast, the CNS neural stem cell is "multipotent" because its progeny have multiple differentiative pathways.

A "neural progenitor cell" is an undifferentiated cell derived from a multipotent self-renewing CNS neural stem cell, and is not itself a stem cell. Some progenitor cells can produce progeny that are capable of differentiating into more than one cell type. For example, an O-2A cell is a glial progenitor cell that gives rise to oligodendrocytes and type II astrocytes, and thus could be termed a "bipotential" progenitor cell. A distinguishing feature of a progenitor cell is that, unlike a stem cell, it has limited proliferative ability and thus does not exhibit self maintenance. It is committed to a particular path of differentiation and will, under appropriate conditions, eventually differentiate into glia or neurons.

The term "precursor cells" refers to the progeny of multipotent self-renewing CNS neural stem cells, and thus includes both progenitor cells and daughter multipotent self-renewing CNS neural stem cells.

Multipotent self-renewing CNS neural stem cells can be obtained from embryonic, post-natal, juvenile or adult neural tissue. The preferred source neural tissue is from mammals, preferably rodents (e.g., mice and rats) and primates, and most preferably, from humans. Method for the isolation, proliferation, and passaging of multipotent self-renewing CNS neural stem cells from adult human neural tissue, embryonic human neural tissue, adult monkey (*Rhesus*) neural tissue, mouse embryonic neural tissue, and juvenile and adult mouse brain tissue, including the establishment of neural stem cells in culture from CNS neural stem cells as well as the differentiation of the CNS neural stem cell progeny, are provided by Weiss *et al.*, United States patents 5,750,376 and 5,851,832 (each incorporated herein by reference). In the method of the present invention however, neurospheres are collagenase-treated to dissociate the aggregated cells, rather than triturated or trypsinized according to the methods used by Weiss *et al.*, United States patents 5,750,376 and 5,851,832 and Johe, United States patent 5,753,506 (each incorporated herein by reference).

Multipotent self-renewing CNS neural stem cells can be obtained from donor tissue by dissociation of individual cells from the connecting extracellular matrix of the tissue, as described by Weiss *et al.*, United States patents 5,750,376 and 5,851,832 and Johe, United States patent 5,753,506. Tissue is removed from a neural region using a sterile procedure, and the cells are dissociated in tissue culture medium using any method known in the art including treatment with enzymes such as trypsin, collagenase and the like, or by using physical methods of dissociation such as with a blunt instrument, as described by Weiss *et al.*, United States patents 5,750,376 and 5,851,832. Dissociated cells are centrifuged at low speed, between 200 and 2000 rpm, usually between 400 and 1000 rpm, and then resuspended in culture medium. The neural cells can be cultured in suspension or on a fixed substrate. Cell suspensions are seeded in any receptacle capable of sustaining cells, particularly culture flasks, culture plates or roller bottles, and more particularly in small culture flasks such as 25 cm² culture flasks. Cells cultured in suspension are resuspended at approximately 5×10^4 to 1×10^6 cells/ml, preferably 1×10^6 cells/ml (for 20 week g.w. tissue). Cells plated on a fixed substrate are plated at approximately 2×10^3 to 10^4 cells/cm², preferably 2.5×10^3 cells/cm².

Collagenase-treated neural stem cell cultures, including the multipotent self-renewing CNS neural stem cells of the neurospheres, can be proliferated either on substrates or in suspension, preferably forming clusters of associated undifferentiated cells, referred to as "neurospheres." After culture in the absence of a substrate, the proliferating neurospheres lift off

the floor of the culture dish and tend to form the free-floating clusters characteristic of neurospheres. The proliferating precursor cells of the neurosphere continue to proliferate in suspension. The neurospheres of the suspension culture can be easily passaged to reinitiate proliferation. In the method of the invention, individual cells in the neurospheres are separated by collagenase treatment. The collagenase-treated neurosphere cells are then replated at the desired density to reinitiate proliferation. Single cells from the dissociated neurospheres are suspended in culture medium containing growth factor, and a percentage of these cells proliferate and form new neurospheres largely composed of undifferentiated cells. This manufacture process can be repeated to result in a logarithmic increase in the number of viable cells at each passage. The procedure is continued until the desired number of cells is obtained.

Weiss *et al.*, United States patents 5,750,376 and 5,851,832 disclose "culture medium containing one or more predetermined growth factors effective for inducing multipotent neural stem cell proliferation." However, different basal media can be used, including, but not limited to:

D-MEM/F12 (Gibco BRL, Gaithersburg, MD);
Ex Vivo 15 (Bio Whittaker, Walkersville, MD);
Neural progenitor basal media, (Clonetics, San Diego, CA); or
combination of the basal media listed above.

The culture medium is supplemented with at least one proliferation-inducing growth factor. As used herein, the term "growth factor" refers to a protein, peptide or other molecule having a growth, proliferative, differentiative, or trophic effect on neural stem cells and/or neural stem cell progeny. Growth factors which may be used for inducing proliferation include any trophic factor that allows neural stem cells and precursor cells to proliferate, including any molecule which binds to a receptor on the surface of the cell to exert a trophic, or growth-inducing effect on the cell. Preferred proliferation-inducing growth factors include members of the EGF superfamily, FGF superfamily, and TGF α superfamily, such as EGF, amphiregulin, acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), transforming growth factor alpha (TGF α), leukocyte inhibitory factor (LIF), glycostatin C and combinations thereof. A preferred combination of proliferation-inducing growth factors is EGF or TGF α with FGF-1 or FGF-2. Growth factors are usually added to the culture medium at concentrations ranging between about 1 fg/ml to 1 mg/ml. Concentrations between about 1 to

100 ng/ml are usually sufficient. Simple titration experiments can easily be performed to determine the optimal concentration of a particular growth factor.

The optimization of media formulation permits a higher percentage of neurospheres initiated from primary brain tissue to be established. We prefer Ex Vivo 15 media. The optimization of media formulation also permits a more consistent growth of neurospheres. To maximize neurosphere development, the collagenase-treated neurosphere cells are typically cultured in the presence of LIF, bFGF, EGF, and neural survival factor, NSF (Cat. CC-4323, Clonetics, San Diego, CA).

A typical media formulation to culture human neural stem cell cultures is provided in

TABLE I.

TABLE I	
Serum-Free N2/EGF Supplemented Culture Medium For Neural Stem Cell Cultures	
Quantity	Reagents
87 ml	DMEM/F12 (Gibco lot. 1012915; Cat. No. 11330-032)
1 ml	N-2 Supplement (Gibco lot 1017018; Cat. No. 17502-014)
1 ml	0.2 mg/ml heparin (Sigma lot 28H0320; Cat. No. H-3149)
1 ml	0.2 M Glutamine (JCR lot 7N2320; Cat. No. 59202-77p)
10 ml	3 % Glucose (Sigma, lot 37H0841; Cat. No. G-7021)
20 µl	100 µg/ml EGF (R&D lot CE107091; Cat. No. 236-EG)
100 µl	20 µg/ml FGF-2 (Gibco lot KCQ411; Cat. No. 13256-029)
100 µl	10 µg/ml LIF (R&D lot OX038021; Cat. No. 250-L)

EGF is added to 100 ml base medium for human neural stem cell cultures after filtering the medium. EGF is relatively stable in the medium. FGF-2 and LIF are added when medium is ready to use. The final concentrations of the supplement reagents are:

TABLE 2

5 µg/ml	Insulin
100 µg/ml	Human transferrin
6.3 ng/ml	Progesterone
16.1 µg/ml	Putrescine
5.2 ng/ml	Selenite
20 ng/ml	EGF
20 ng/ml	FGF-2
10 ng/ml	LIF
2 µg/ml	heparin
2 mM	L-glutamine
6 mg/ml	Glucose

Collagenase-treated neural stem cell cultures can also be differentiated using the differentiation paradigms as described in Weiss *et al.*, United States patents 5,750,376 and 5,851,832. For example, (1) collagenase-treated neural stem cell cultures can be differentiated by a rapid differentiation after being plated on poly-L-ornithine-coated glass coverslips in medium containing 0.5% fetal bovine serum (FBS); (2) collagenase-treated neural stem cell cultures can be differentiated using dissociated neurospheres in EGF-free complete medium containing 1% FBS; (3) collagenase-treated neural stem cell cultures can be differentiated using single neurospheres plated onto laminin-coated glass coverslips; (4) collagenase-treated neural stem cell cultures can be differentiated using single dissociated neurospheres, collagenase-treated, and plated onto a 35 mm culture dish; (5) collagenase-treated neural stem cell cultures can be differentiated using neurospheres co-cultured with striatal astrocytes. In a preferred method of differentiation, neurosphere cells are plated on a laminin coated substrate in the presence of FBS. The resulting differentiated cells are probed by indirect immunocytochemistry for the presence of neuron, astrocytes and oligodendrocytes, for example, using antibodies to MAP-2, tau-1, neurofilament 168 kDa, β -tubulin, GABA, substance P (neuronal markers), GFAP (astrocytic marker), O4, and MBP (oligodendrocyte markers). All three neural cell types are expected to be identified.

Genetic Modification of Collagenase-treated Neural Stem Cell Cultures. The neural stem cell cultures described herein may be genetically modified according to any suitable method known in the art, including *in vitro* genetic modification, or generation of genetically modified neural stem cell cultures from transgenic mammals. The genetic modification of neural stem cells is performed either by infection with recombinant retroviruses or transfection using

methods known in the art (see, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, N.Y., 1989). Methods of making genetically modified neural stem cells cultures are described, e.g., Weiss, United States patent 5,750,376, incorporated herein by reference.

Generally, the term "genetic modification" refers to the stable or transient alteration of the genotype of a precursor cell by intentional introduction of exogenous DNA. DNA may be synthetic, or naturally derived, and may contain genes, portions of genes, or other useful DNA sequences. The term "genetic modification" includes various gene activation methods known in the art. See, e.g., United States patents 5,733,761 and 5,733,746, each herein incorporated by reference.

In certain embodiments the neural stem cells are genetically modified to produce a biologically active molecule, including hormones, enzymes, neurotransmitters, antibodies, cytokines, lymphokines, growth factors, trophic factors, or biological response modifiers. Alternatively the neural stem cells are genetically modified to provide a metabolic or immunologic function upon implantation in a host, preferably a human. It may also be desired to genetically modify cells so they secrete a certain growth factor product. The term "growth factor product" refers to a protein, peptide, mitogen, or other molecule having a growth, proliferative, differentiative, or trophic effect (e.g., NGF, BDNF, the neurotrophins, CNTF, amphiregulin, FGF-1, FGF-2, EGF, TGF α , TGF β s, PDGF, IGFs, and the interleukins). Neurosphere progeny cells can also be modified to express a certain growth factor receptor (e.g., p75 low affinity NGF receptor, CNTF receptor, the *trk* family of neurotrophin receptors, EGF-R, FGF-R, and amphiregulin receptors). Collagenase-treated neural stem cell cultures can be engineered to produce various neurotransmitters, neurotransmitter receptors, or neurotransmitter-synthesizing enzymes.

Transplantation of Neural Stem Cell Cultures to Alleviate Human Disorders.

Collagenase-treated neural stem cell cultures can be produced and transplanted into mammalian hosts, preferably human patients, for the treatment of various disorders, both in the central nervous system ("CNS") and systemically. Cells are delivered to the subject by any suitable means known in the art. If delivered to the central nervous system, then the cells are administered to a particular region using any method which maintains the integrity of surrounding areas of the brain, preferably by injection cannula. Injection methods exemplified by those used by Duncan *et al.*, 17 J. Neurocytology 351-361 (1988), and scaled up and modified for use in humans are

preferred. Methods for the injection of cell suspensions such as fibroblasts into the CNS may also be employed for injection of neural precursor cells. Additional approaches and methods may be found in *Neural Grafting in the Mammalian CNS*, Bjorklund & Stenevi, eds. (1985).

Collagenase-treated neural stem cell cultures can be produced and transplanted using the above procedures to treat various neurodegenerative disorders. Such CNS disorders encompass numerous afflictions such as neurodegenerative diseases (e.g. Alzheimer's and Parkinson's), acute brain injury (e.g. stroke, head injury, cerebral palsy) and a large number of CNS dysfunctions (e.g. depression, epilepsy, and schizophrenia). In recent years neurodegenerative disease has become an important concern due to the expanding elderly population which is at greatest risk for these disorders. These diseases, which include Alzheimer's Disease, Multiple Sclerosis (MS), Huntington's Disease, Amyotrophic Lateral Sclerosis, and Parkinson's Disease, have been linked to the degeneration of neural cells in particular locations of the CNS, leading to the inability of these cells or the brain region to carry out their intended function. By providing for maturation, proliferation and differentiation into one or more selected lineages through specific different growth factors the progenitor cells may be used as a source of committed cells. In one series of embodiments, collagenase-treated neural stem cell cultures can be produced and transplanted using the above procedures for the treatment of demyelination diseases. Any suitable method for the implantation of cells near to the demyelinated targets may be used so that the cells can become associated with the demyelinated axons.

Neural stem cell cultures made according to the present invention may also be used to produce a variety of blood cell types, including myeloid and lymphoid cells, as well as early hematopoietic cells (see, Bjornson *et al.*, 283 SCIENCE 534 (1999), incorporated herein by reference).

In Vitro Models of CNS Development, Function and Dysfunction, and Methods for Screening Effects of Drugs on Cells. Collagenase-treated neural stem cell cultures cultured *in vitro* can be used for the screening of potential neurologically therapeutic compositions. These compositions can be applied to cells in culture at varying dosages, and the response of the cells monitored for various time periods. Physical characteristics of the cells can be analyzed by observing cell and neurite growth with microscopy. The induction of expression of new or increased levels of proteins such as enzymes, receptors and other cell surface molecules, or of neurotransmitters, amino acids, neuropeptides and biogenic amines can be analyzed with any technique known in the art which can identify the alteration of the level of such molecules. These

techniques include immunohistochemistry using antibodies against such molecules, or biochemical analysis. Such biochemical analysis includes protein assays, enzymatic assays, receptor binding assays, enzyme-linked immunosorbent assays (ELISA), electrophoretic analysis, analysis with high performance liquid chromatography (HPLC), Western blots, and radioimmune assays (RIA). Nucleic acid analysis such as Northern blots can be used to examine the levels of mRNA coding for these molecules, or for enzymes which synthesize these molecules. Alternatively, cells treated with these pharmaceutical compositions can be transplanted into an animal, and their survival, ability to form neuronal connections, and biochemical and immunological characteristics examined as previously described.

The collagenase-treated neural stem cell cultures can be used in methods of determining the effect of a biological agents on neural cells. The term "biological agent" refers to any agent, such as a virus, protein, peptide, amino acid, lipid, carbohydrate, nucleic acid, nucleotide, drug, pro-drug or other substance that may have an effect on neural cells whether such effect is harmful, beneficial, or otherwise. Biological agents that are beneficial to neural cells are referred to herein as "neurological agents", a term which encompasses any biologically or pharmaceutically active substance that may prove potentially useful for the proliferation, differentiation or functioning of CNS cells or treatment of neurological disease or disorder. To determine the effect of a potential biological agent on neural cells, a culture of collagenase-treated neural stem cell cultures is obtained and proliferated *in vitro* in the presence of a proliferation-inducing growth factor. Generally, the biological agent will be solubilized and added to the culture medium at varying concentrations to determine the effect of the agent at each dose. The culture medium may be replenished with the biological agent every couple of days in amounts, so as to keep the concentration of the agent somewhat constant.

Thus, it is possible to screen for biological agents that increase the proliferative ability of progenitor cells which would be useful for generating large numbers of cells for transplantation purposes. It is also possible to screen for biological agents which inhibit precursor cell proliferation, using collagenase-treated neural stem cell cultures. Also, the ability of various biological agents to increase, decrease or modify in some other way the number and nature of differentiated neural cells can be screened on collagenase-treated neural stem cell cultures that have been induced to differentiate. The effects of a biological agent or combination of biological agents on the differentiation and survival of differentiated neural cells can then be determined. It

is also possible to determine the effects of the biological agents on the differentiation process by applying them to collagenase-treated neural stem cell cultures prior to differentiation.

Other features, objects, and advantages of the invention will be apparent from the description and from the claims. In the specification and the appended claims, the singular forms include plural referents unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All patents and publications cited in this specification are incorporated by reference. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. These examples should in no way be construed as limiting the scope of the invention, as defined by the appended claims.

EXAMPLE 1 COLLAGENASE PROTOCOL

1. Rinse tissue several times with Ca^{++} , Mg^{++} -free phosphate buffered saline (PBS).
2. In a Petri dish, cut tissue into 1-2 mm cubed pieces using crossed scalpels. Pour the tissue (packed volume ~7.5 ml) and PBS (~8 ml) into a 50 ml centrifuge tube. Rinse the dish with PBS as necessary to remove tissue, and transfer to the centrifuge tube.
3. Centrifuge the tubes gently to sediment the tissue and free cells (<1000 rpm).
4. Remove the supernatant carefully using a vacuum line.
5. For up to 1.0 g of tissue, add 5.0 ml 0.1% Collagenase, 0.1% Hyaluronidase in HBSS without Ca^{++} , Mg^{++} which contains 1.0% bovine serum albumin (BSA).
6. Incubate in 37°C waterbath with occasional light agitation for 1 hour. At the end of 1 hr, vortex for approximately 3 sec, and evaluate the extent tissue dissociation. If large pieces of intact tissue remains, continue incubation at 37°C for another 30-45 min. Stand the tube upright for 1-2 min to allow large cell aggregates to settle out. Transfer the supernatant to a fresh tube (1).
7. Top up the fresh tube (1) with PBS + 0.1% BSA and centrifuge at approximately 900 rpm for 6 min. Remove the supernatant, which should be cloudy with debris, to a fresh 50 ml centrifuge tube (2). Recentrifuge both tubes (1) and (2) at 900 rpm.

8. Discard the supernatant from tube (1) and examine the pellet from tube (2) for viable cells. If worthwhile, combine the pelleted cells from tube (2) with the contents of tube (1). Fill up the tube with PBS + 0.1% BSA and respin for 6 min at 900 rpm, discarding the supernatant when finished. Resuspend the cells in PBS and count with trypan blue. At this stage, the cell suspension should be relatively free of debris, and should consist predominantly of healthy cells.

EXAMPLE 2 COLLAGENASE RESULTS

Collagenase treatment provided increased number of viable neural stem cells using the collagenase method. Cells are counted in trypan blue on hemocytometer. A raw count is the number of live cells in defined area.

TABLE 3

	Live	Dead	Cells/ml	Total cells	% viability
Collagenase	121	12	8.07×10^5	8.07×10^6	91
Trituration	45	49	3×10^5	3×10^6	48

Collagenase treatment provided increased number of proliferated neural stem cells over time using the collagenase method.

TABLE 4

Days	Trituration	Collagenase Treatment	% Viability Trituration	% Viability Collagenase
0	2.00×10^6	2.00×10^6		
7	4.60×10^6	1.60×10^7	40.00%	89%
17	1.04×10^7	7.28×10^7	52.00%	93%
25	8.80×10^6	1.67×10^8	37.00%	78%
33	1.55×10^7	6.75×10^8	48.00%	91%
41	2.33×10^7	2.61×10^9	38.00%	98%
			Average	Average
			43.00%	90.0%
			$\pm 6.63\%$	$\pm 7.4\%$

TABLE 5						
Days	Trituration	Input Cell No.	Output Cell No.	% Viability	X Expansion	Accumulated Cell No.
0	P24-P1	2.00×10^6				2.00×10^6
7	P25-P2	2.00×10^6	4.60×10^6	40%	2.3	4.60×10^6
17	P26-P3	2.00×10^6	4.50×10^6	52%	2.3	1.04×10^7
25	P27-P4	2.00×10^6	1.70×10^6	37%	0.9	8.80×10^6
33	P28-P5	1.95×10^6	3.00×10^6	48%	1.8	1.55×10^7
41	P29-P6	2.00×10^6	3.00×10^6	38%	1.5	2.33×10^7
				Average		
				43%		

TABLE 6						
Days	Collagenase	Input Cell No.	Accumulated Cell No.	% Viability	X Expansion	Accumulated Cell No.
0	P24-P1	2.00×10^6				2.00×10^6
7	P25-P2	2.00×10^6	1.60×10^7	89%	8.0	1.60×10^7
17	P26-P3	2.00×10^6	9.70×10^6	93%	4.6	7.28×10^7
25	P27-P4	2.00×10^6	4.60×10^6	78%	2.3	1.67×10^8
33	P28-P5	1.70×10^6	8.06×10^6	91%	4.0	7.94×10^8
41	P29-P6	2.00×10^6	5.73×10^6	98%	3.9	2.27×10^9
				Average		
				90%		

The foregoing description has been presented only for the purposes of illustration and is not intended to limit the invention to the precise form disclosed, but by the claims appended hereto.

ART 34

CLAIMS

1. A method for the *in vitro* proliferation of a neural stem cell culture comprising the steps of:
 - (a) obtaining dissociated neural tissue in cell suspension, the suspension containing one or more multipotent neural stem cells capable of producing progeny that are capable of differentiating into neurons and glia;
 - (b) culturing the cell suspension in a culture medium containing at least one proliferation-inducing growth factor to proliferate the neural stem cells in (a) to generate a neural stem cell culture; and
 - (c) passaging the cell culture in (b) by treating the culture with an amount of a collagenase preparation effective to dissociate the cells in the culture and passing the cell culture to a culture medium containing at least one proliferation-inducing growth factor to further proliferate the neural stem cell culture.
2. The method of claim 1 wherein the amount of the collagenase preparation is between 18-180 mU/ml.
3. The method of claim 1 wherein the amount of the collagenase preparation is between 54-126 mU/ml.
4. The method of claim 1 wherein the collagenase preparation further comprises at least one molecule selected from the group consisting of sulfhydryl protease, clostripain, aminopeptidase, or combinations thereof.
5. The method of claim 1 wherein the collagenase preparation is substantially pure, and contains minimal secondary proteolytic activity.
6. The process of claim 1, wherein the proliferation-inducing growth factor is selected from the group consisting of epidermal growth factor, amphiregulin, acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor alpha, leukocyte inhibitory factor (LIF), glycostatin C and combinations thereof.

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7. The method of claim 1 wherein the neural stem cell culture comprises genetically modified neural stem cells.
8. The method of claim 1, further comprising the step of differentiating the neural stem cell culture of (c) to produce a cell culture comprising differentiated neural cells selected from the group consisting of astrocytes, neurons, oligodendrocytes, and combinations thereof.
9. The method of claim 1 or claim 8, further comprising contacting the neural stem cell culture with a biological agent, and determining the effects of the biological agent on cells in the culture.
10. The method of claim 1 wherein the neural stem cell culture is a suspension culture.
11. The method of claim 1 wherein the neural stem cell culture is an adhesion culture.
12. The method of claim 1 wherein the neural stem cell culture comprises human neural stem cells.
13. A method for the *in vitro* proliferation of a neural stem cell culture wherein the percent viability of the cells in the culture is at least 60%, the method comprising the steps of:
 - (a) obtaining dissociated neural tissue in cell suspension, the suspension containing one or more multipotent neural stem cells capable of producing progeny that are capable of differentiating into neurons and glia;
 - (b) culturing the cell suspension in a culture medium containing at least one proliferation-inducing growth factor to proliferate the neural stem cells in (a) to generate a neural stem cell culture; and
 - (c) passaging the cell culture in (b) by treating the culture with an amount of a collagenase preparation effective to dissociate the cells in the culture and passing the cell culture to a culture medium containing at least one proliferation-inducing growth factor to further proliferate the neural stem cell culture.

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14. The method of claim 13, wherein the percent viability of the cells in the culture is at least 75% after being passaged.
15. The method of claim 13, wherein the percent viability of the cells in the culture is at least 85% after being passaged.
16. The method of claim 13, wherein the amount of the collagenase preparation is between 18-180 mU/ml.
17. The method of claim 13, wherein the amount of the collagenase preparation is between 54-126 mU/ml.
18. The method of claim 13, wherein the collagenase preparation further comprises at least one molecule selected from the group consisting of sulphhydryl protease, clostripain, aminopeptidase, or combinations thereof.
19. The method of claim 13, wherein the collagenase preparation is substantially pure, and contains minimal secondary proteolytic activity.
20. The method of claim 13, wherein the neural stem cell culture comprises genetically modified neural stem cells.
21. The method of claim 13, further comprising the step of differentiating the neural stem cell culture of (c) to produce a cell culture comprising differentiated neural cells selected from the group consisting of astrocytes, neurons, oligodendrocytes, and combinations thereof.
22. The method of claim 13 wherein the neural stem cell culture is a suspension culture.
23. The method of claim 13 wherein the neural stem cell culture is an adhesion culture.
24. The method of claim 13 wherein the neural stem cell culture comprises human neural stem cells.

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ART 32

A method for expanding a neural stem cell culture wherein there is at least a three-fold increase in cell number between weekly passages, the method comprising the steps of:

- (a) culturing a neural stem cell culture that contains one or more multipotent neural stem cells capable of producing progeny that are capable of differentiating into neurons and glia in a culture medium containing at least one proliferation-inducing growth factor to proliferate the culture; and
- (b) passaging the cell culture in (a) by treating the culture with an amount of a collagenase preparation effective to dissociate the cells in the culture and passing the cell culture to a culture medium containing at least one proliferation-inducing growth factor to further proliferate the neural stem cell culture.

- 26. The method of claim 25 wherein there is at least a five-fold increase in cell number between weekly passages.
- 27. The method of claim 25 wherein the neural stem cell culture is a suspension culture.
- 28. The method of claim 25 wherein the neural stem cell culture is an adhesion culture.
- 29. The method of claim 25 wherein the neural stem cell culture comprises human neural stem cells.
- 30. A method for expanding a neural stem cell culture wherein the accumulated cell number can be expanded from 10^6 cells to at least 10^9 cells in less than 49 days, the method comprising the steps of:
 - (a) culturing a neural stem cell culture that contains one or more multipotent neural stem cells capable of producing progeny that are capable of differentiating into neurons and glia in a culture medium containing at least one proliferation-inducing growth factor to proliferate the culture; and
 - (b) passaging the cell culture in (a) by treating the culture with an amount of a collagenase preparation effective to dissociate the cells in the culture and passing the cell culture to a culture medium containing at least one proliferation-inducing growth factor to further proliferate the neural stem cell culture.

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31. The method of claim 30 wherein the accumulated cell number can be expanded from 10^6 cells to at least 10^9 cells in less than 42 days.
32. The method of claim 30 wherein the neural stem cell culture is a suspension culture.
33. The method of claim 30 wherein the neural stem cell culture is an adhesion culture.
34. The method of claim 30, wherein the neural stem cell culture comprises human neural stem cells.

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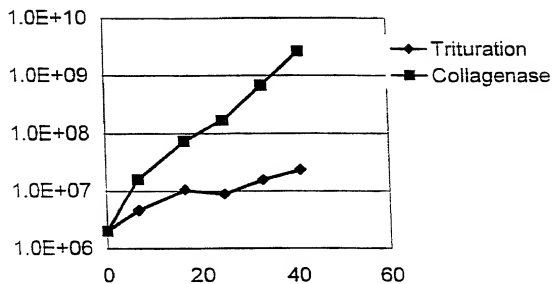


Fig. 1

**COMBINED DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor of the subject matter (an original, first and joint inventor) which is claimed and for which a utility patent is sought on the invention entitled:

**USE OF COLLAGENASE IN THE PREPARATION OF NEURAL STEM CELL
CULTURES**

the specification of which:

☒ was filed on July 31, 2001, with the United States Patent and Trademark Office and received International Application Number PCT/US00/05402, said application also bearing Attorney Docket No. 17810-509 NATL.

☐ is attached hereto.

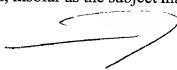
I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

☐ I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application designating at least one country other than the United States listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Appln. Number	Country (if PCT, so indicate)	Filing Date (dd/mm/yy)	Priority Claimed	
			Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>

☒ I hereby claim the benefit under Title 35, United States Code, § 119(e) or §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter



of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

Application No. (U.S.S.N.)	Filing Date (dd/mm/yy)	Status (Patented, Pending, Abandoned)
09/258,529	26 February 1999	Pending

PCT International Applications designating the United States:

PCT Appln No.	US Serial No.	PCT Filing Date	Status
PCT/US00/05402	09/890,539	February 25, 2000	Pending

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

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